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Our goal is to understand the role of a novel epithelium-specific Ets transcription factor, PDEF, in prostate cancer. Our progress over the last year has provided significant further evidence that PDEF is an important player in prostate cancer. We were able to demonstrate that PDEF expression is regulated by androgen and that PDEF is indeed a critical regulator of PSA gene expression in prostate cancer cells. We, furthermore, demonstrated that PDEF is significantly upregulated in a number of prostate cancer patients and, thus, possibly may serve as a marker for prostate cancer. We also showed that PDEF expression is downregulated by the growth inhibitor TGF-β1 and that PDEF itself can regulate cell proliferation. We also identified a variety of target genes for PDEF using transcriptional profiling which will further help us to understand the biological function of PDEF. Our results as well as the critical roles of other Ets factors in cellular differentiation and tumorigenesis strongly suggest that PDEF is an important regulator of prostate gland development and plays a role in prostate and breast cancer progression or development. The new data have further strengthened our believe that PDEF is a prime target for drug development.

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a. Introduction

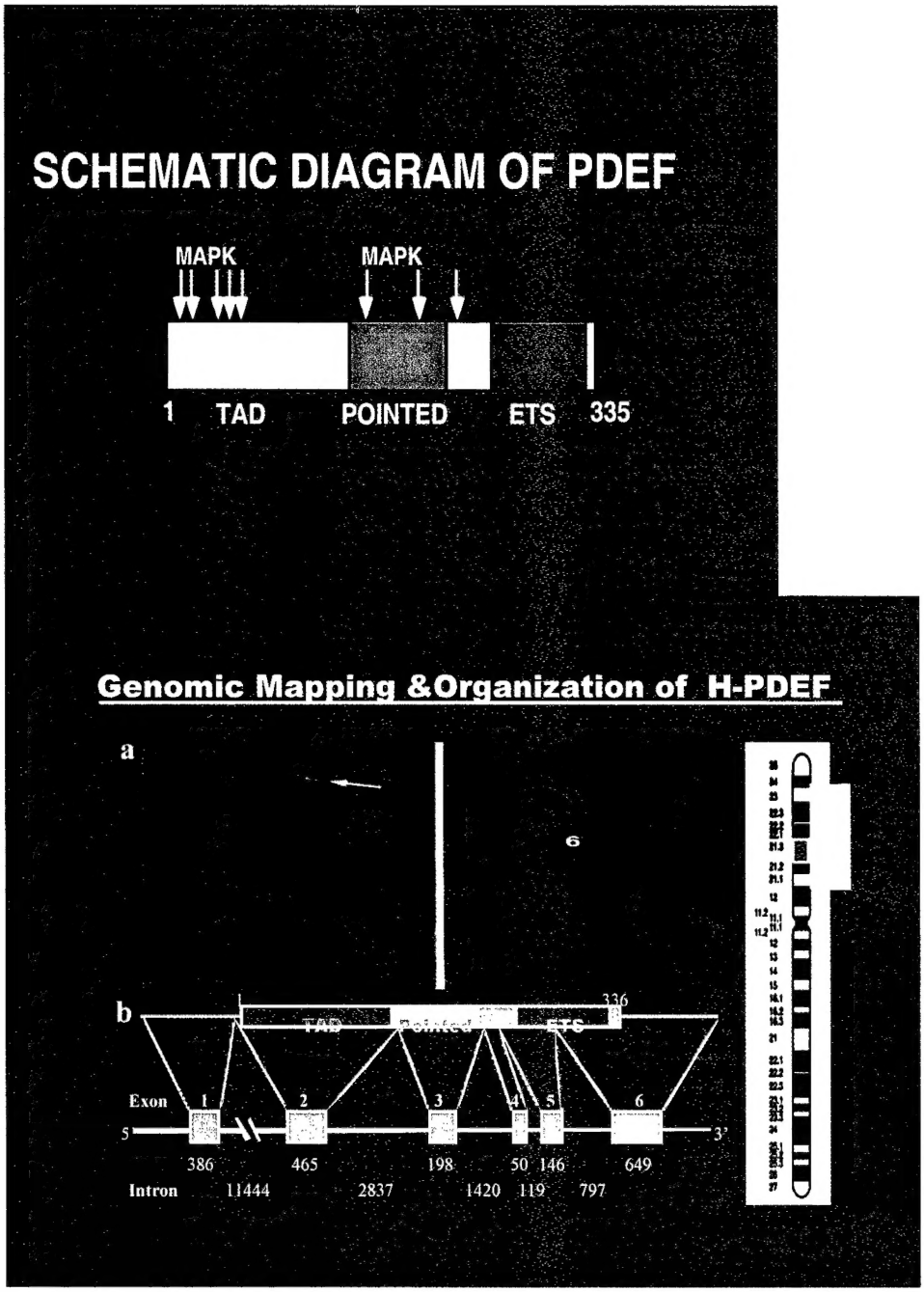
Prostate cancer has become the most common solid cancer in older men and is one of the most frequent causes of cancer deaths. Although androgen ablation therapy, surgery and radiation therapy are effective for the treatment of local prostate cancer, there is no effective treatment available for patients with metastatic androgen-independent disease. The poor prognosis for androgen-independent advanced prostate cancer reflects in part the lack of knowledge about the tumor's basic biology, although progress has been made in identifying defects of various oncogenes and tumor suppressor genes. In particular, very little is known about the molecular mechanisms that trigger the conversion of an initially androgen-dependent cancer to androgen-independence. Our goal is to understand the role of a novel prostate epithelium-specific transcription factor, PDEF, a member of the Ets transcription factor/oncogene family in human prostate cancer that uniquely among the Ets family prefers binding to a GGAT rather than a GGAA core. PDEF is expressed in the luminal epithelial cells of normal human prostate and PDEF expression is significantly elevated in cancerous portions of the prostate. PDEF acts as an androgen-independent transcriptional activator of the PSA promoter, a diagnostic marker used for monitoring androgen-dependent and –independent prostate cancer. PDEF also directly interacts with the DNA binding domain of the androgen receptor and with the prostate-specific homeobox gene NKX3.1 and enhances androgen-mediated activation of the PSA promoter. Thus, our hypothesis is that PDEF bypasses or activates the androgen receptor and thereby contributes to the progression from an initially androgen-dependent prostate cancer to an androgen-independent cancer. We propose to determine the role of this novel member of the Ets family in the conversion of prostate cancer to androgen independence. Our results as well as the critical roles of other Ets factors in cellular differentiation and tumorigenesis strongly suggest that PDEF is an important regulator of prostate gland development and plays a role in prostate epithelial cell transformation and/or prostate cancer progression. Our long term goal is to explore the possibility to use this new factor as another diagnostic tool and as a potential therapeutic target for prostate cancer.

b. Body

In the last year we made significant progress in several specific aims. Following is a summary of the progress made during this funding period.

Chromosomal mapping and genomic organization of the human PDEF gene and identification of a functional promoter

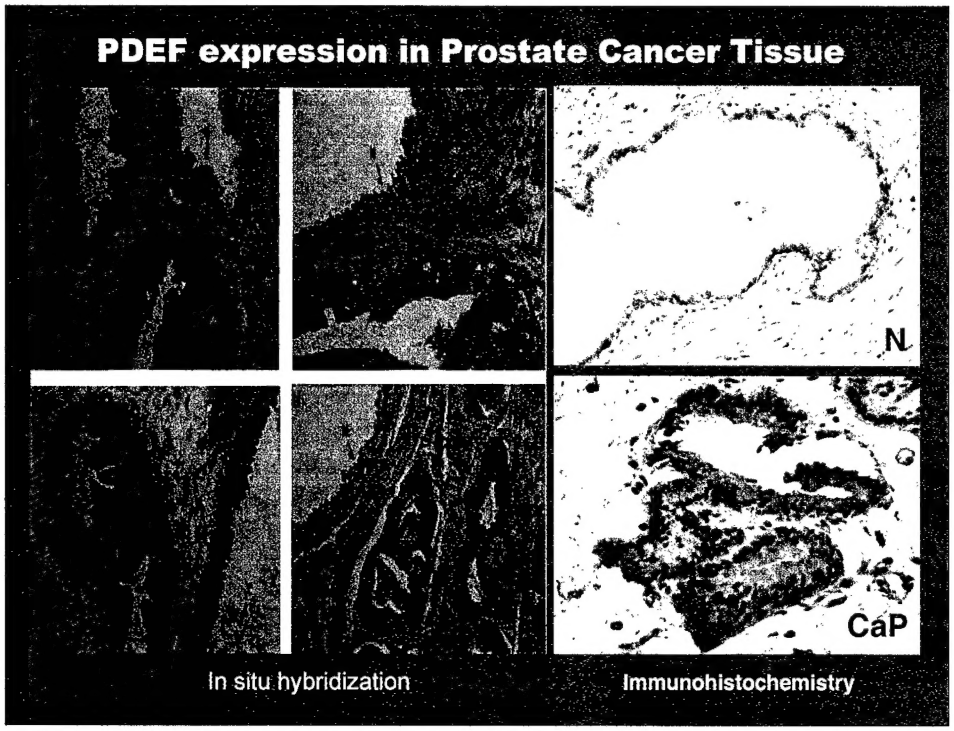
PDEF is primarily expressed in prostate epithelial cells and other hormone regulated epithelia. PDEF regulates the expression of PSA, a widely used diagnostic marker for prostate cancer, in both an androgen dependent and independent manner, and directly interacts with the DNA binding domain of the androgen receptor. Due to the important role that other Ets factors play in cellular differentiation and human cancer, PDEF is expected to be a critical regulator of epithelial gene expression and transformation. We report here the chromosomal mapping, the structural organization and identification of a functional promoter of the human PDEF gene. The human PDEF gene is positioned within the MHC cluster region on chromosome 6p21.3 and contains 6 exons, which span approximately 18.5kb of genomic DNA. Analysis of the immediate promoter region of the human PDEF genes demonstrates the presence of a TATA box as well as potential binding sites for Ets factors, AP-1, and an androgen response element. Transfection experiments demonstrate that a 2.5 kb fragment of the 5' upstream region acts as a strong promoter in a subset of epithelial cell lines.

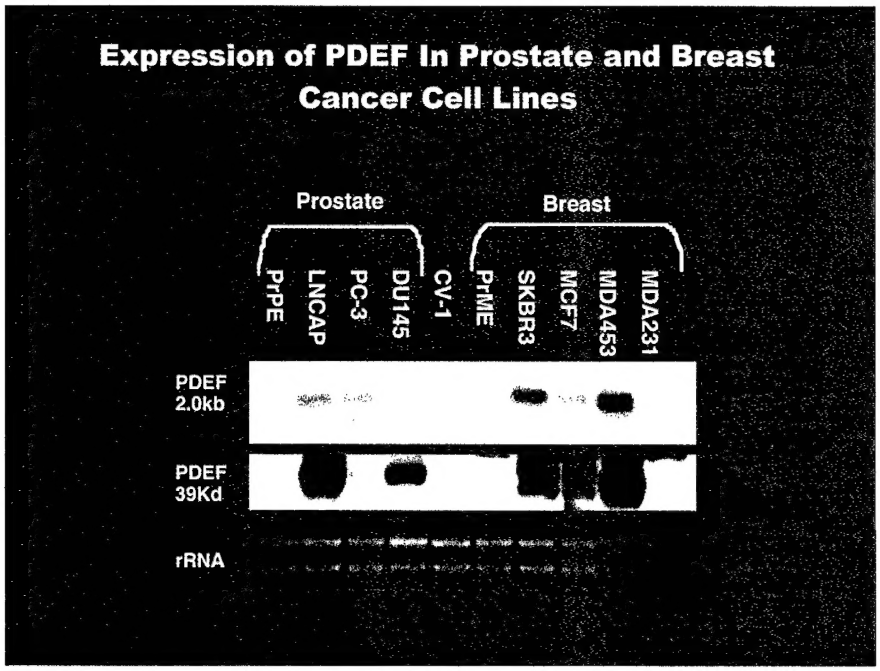


PDEF expression is strongly enhanced in human prostate cancer

To determine whether PDEF transcripts are expressed in human prostate and to localize PDEF mRNA expression in prostate cancer, we performed in situ hybridization and immunohistochemistry on paraffin sections of primary prostate cancer tissues and normal prostate using a digoxygenin labeled PDEF antisense probe for in situ hybridization and a polyclonal antibody against PDEF for immunohistochemistry. Strikingly, only the transformed epithelial cells stained strongly for PDEF mRNA or protein. Only weak staining was observed in normal prostate epithelium. PDEF protein expression was observed in both cytoplasm and the nucleus. Tumor tissue did not stain uniformly, but showed striking differences in different parts of tumorsa and in different patient samples. Thus, PDEF expression might be a marker for prostate cancer development and/or progression and could be used as a diagnostic or possibly prognostic marker in prostate biopsies. Northern and RT-

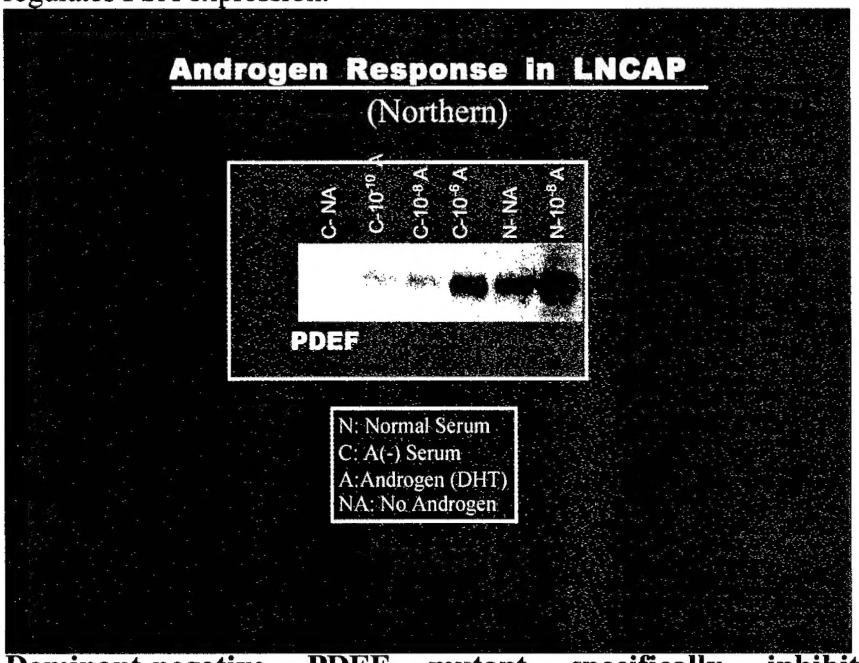
PCR analysis showed that prostate cancer cell lines LNCaP and PC-3 express high levels of PDEF, whereas primary prostate epithelial cells do not. In addition to prostate cancer, several breast cancer cell lines also have very high PDEF expression, even though the normal counterpart does not express PDEF. These results indicate overexpression of PDEF might be a biomarker for certain types of cancer that are possibly responsive to androgen or steroids. Interestingly, immunohistochemistry on tissue arrays representing all major human organs and tissue types demonstrated that PDEF is only highly expressed in proliferative endometrium, but not in glandular endometrium or other tissues. This finding suggests that PDEF may play a role in endometrium proliferative processes and, thus, may be involved in endometriosis.





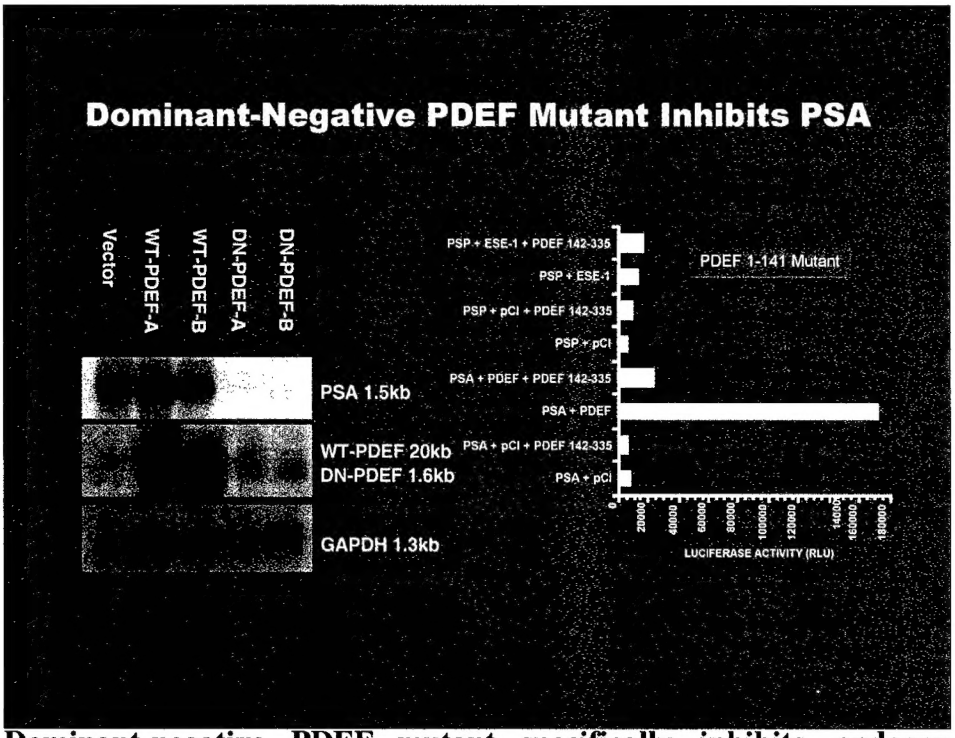
PDEF expression is induced by androgen

Androgen is a critical regulator for prostate cell growth and PSA expression. Since PDEF is highly expressed in prostate cancer cells and transactivates the PSA promoter, PDEF might be one of the targets for androgen induced prostate growth and transformation. To test this, we measured the PDEF transcript levels in response to androgen DHT in LNCaP cells grown in androgen deficient medium. PDEF expression was highly reduced after depletion of androgen and was induced in a dose dependent manner by androgen, indicating PDEF is also involved in androgen mediated molecular responses. Expression of PDEF under these experimental conditions directly correlated with PSA levels, further supporting the hypothesis that PDEF regulates PSA expression.



Dominant-negative PDEF mutant specifically inhibits PDEF mediated transactivation of the PSA promoter

We have generated a dominant-negative deletion mutant of PDEF that lacks the amino-terminal transactivation domain ($PDEF_{\Delta 1\text{-}141}$). This mutant is still able to bind to PDEF binding sites, but does not transactivate. Increasing amounts of an expression vector for this mutant inhibited transactivation of the PSA promoter by wild type PDEF by more than 90% . These data suggest that this PDEF mutant acts as a specific dominant-negative mutant and we have used this mutant to block the activity of endogenous PDEF.



Dominant-negative PDEF mutant specifically inhibits endogenous PSA gene expression in PSA positive LNCaP cells

In order to evaluate whether PDEF is a critical factor for PSA gene expression in vivo we stably transfected PSA positive LNCaP cells with the dominant-negative PDEF mutant, as well as the empty parental expression vector, since LNCaP cells also express endogenous PDEF. Several stably transfected clones were isolated and analyzed by Northern Blotting for expression of the endogenous PSA gene. In contrast to the parental vector control clones that expressed high levels of PSA, clones expressing the dominant-negative PDEF did not express any detectable levels of PSA. These data most vividly demonstrate that PDEF is a critical factor for PSA gene expression and, therefore, may play an important role in prostate cancer.

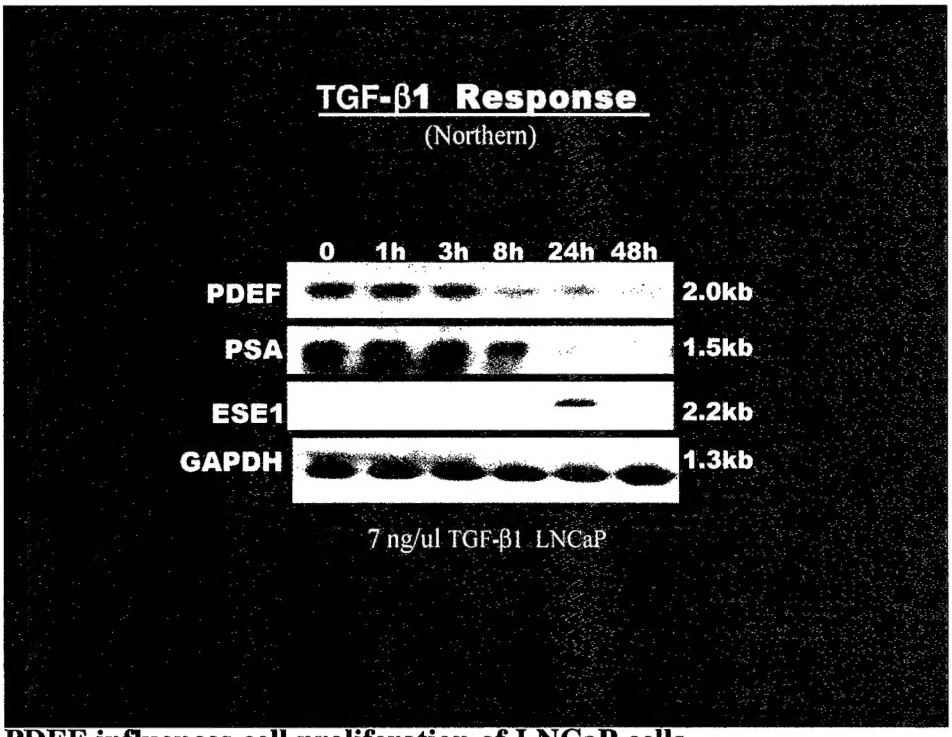
PDEF overexpression induces endogenous PSA gene expression in LNCaP cells

We transiently transfected LNCaP cells with an expression vector encoding PDEF to determine whether PDEF overexpression has an effect on endogenous PSA gene expression. Indeed PDEF overexpression significantly enhanced endogenous PSA gene expression in LNCaP cells, confirming the data obtained with the dominant-negative mutant PDEF. Together, all these data place PDEF in the center of PSA gene regulation and may suggest that PDEF plays a role in androgen-independent PSA gene expression in hormone-refractory prostate cancer.

TGF-β1 inhibits PDEF in prostate and breast cancer cell lines

Growth regulatory proteins of the transforming growth factor-beta family (TGF-β) are one of the few classes of endogenous inhibitors of cell growth. Contrary to the first notion that these proteins may be downregulated in cancer cells to promote their growth, generally there is a marked increase in the expression of TGF-β in many cancers, including those of the prostate and breast. Furthermore, in many of these cancers high expression correlates with more advanced stages of malignancy and decreased survival. The increased expression of TGF-β is usually accompanied by a loss in the growth inhibitory response to TGF-β. We tested whether PDEF expression is regulated by TGF-β1 and the response correlates with the malignancy. We measured the mRNA levels of PDEF in both breast and prostate cancer cell lines. We found that TGF- β1 treatment reduced PDEF expression in hormone dependent MCF-7 and LNCaP cells but not in more malignant MDA-MB-453 cell line. These findings

further suggest that expression of PDEF is regulated by various cancer growth modulating factors.



PDEF influences cell proliferation of LNCaP cells

Since expression of PDEF was upregulated by the proliferation promoting androgen and downregulated by the growth-inhibitory TGF-β1, we were interested to know whether PDEF is involved in cell proliferation. We tested the stably transfected LNCaP cell clones that either overexpress wild type or dominant-negative mutant PDEF in a proliferation assay and compared the data to LNCaP cells trasnfected with the parental vector. There was a clear difference in cell proliferation of cells expressing the dominant-negative PDEF. These cells had a highly reduced proliferation rate in comparison to the control cells. Wild type PDEF expressing clones had a slightly enhanced proliferation rate when compared to the control cells. These data suggest that one of the functions of PDEF may involve regulation of cell proliferation.

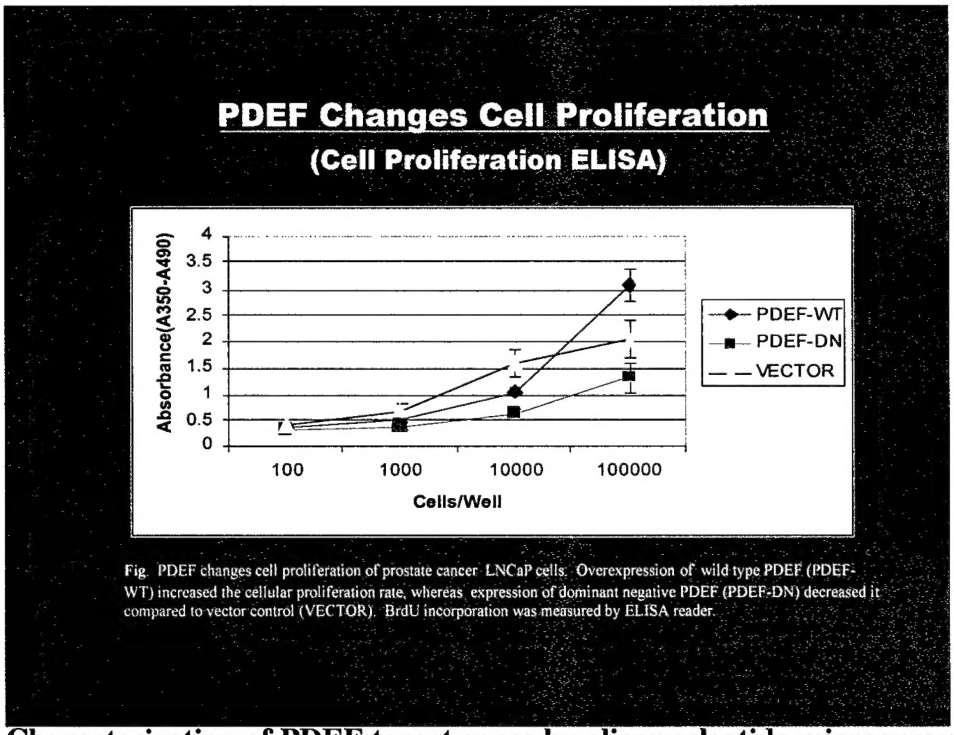


Fig. PDEF changes cell proliferation of prostate cancer LNCaP cells. Overexpression of wild type PDEF (PDEF-WT) increased the cellular proliferation rate, whereas expression of dominant negative PDEF (PDEF-DN) decreased it compared to vector control (VECTOR). BrdU incorporation was measured by ELISA reader.

Characterization of PDEF target genes by oligonucleotide microarrays

We have used Affymetrix oligonucleotide microarrays as well as Clontech Atlas Arrays to determine target genes for PDEF. LNCaP cells as well as 293 cells were transfected for different times with wild type or dominant-negative mutant PDEF or the parental vector. RNA was harvested from duplicate or triplicate experiments and analyzed by hybridization to Affymetrix HU95A chips that contain ~12000 human genes. Using sophisticated novel bioinformatics tools we have obtained a number of genes whose expression is significantly modulated by PDEF. We are now in the process of validating these targets by real-time PCR, Northern blot analysis and cloning of the promoter regions of interesting candidate genes. Among others we found a variety of prostate cancer related genes that are regulated by PDEF.

Development of chromatin immunoprecipitation (CHIP) for determining PDEF target genes in vivo

In order to determine the in vivo targets for PDEF we decided to use the CHIP approach which enables us to cross-link PDEF to the target genes within a living cell. The crosslinked chromatin is then isolated from the cells, sonicated and immunoprecipitated with PDEF specific antibodies. After reversal of the crosslink, bound DNA is eluted and analyzed by PCR or subcloning and sequencing for the presence of the target genes' regulatory regions. In order to establish and test this approach first on a model system, we used antibodies against NF-κB p65 where many target genes have previously been identified and validated. THP-1 cells were stimulated with LPS for different times, crosslinked and chromatin immunoprecipitated using p65 specific antibodies. We analyzed for the presence of several p65 target genes and were able to detect by PCR all of them only in stimulated, but not unstimulated cells and only in the p65 specific immunoprecipitate. We are no applying the same approach using PDEF specific antibodies.

c. Key Research Accomplishments

- Chromosomal mapping and genomic organization of the human PDEF gene and identification of a functional promoter
- PDEF expression is strongly enhanced in human prostate cancer
- PDEF expression is induced by androgen
- Dominant-negative PDEF mutant specifically inhibits PDEF mediated transactivation of the PSA promoter

- Dominant-negative PDEF mutant specifically inhibits endogenous PSA gene expression in PSA positive LNCaP cells
- PDEF overexpression induces endogenous PSA gene expression in LNCaP cells
- TGF-β1 inhibits PDEF expression in prostate and breast cancer cell lines
- PDEF influences cell proliferation of LNCaP cells
- Characterization of PDEF target genes by oligonucleotide microarrays

d. Reportable Outcomes

LNCaP cell clones stably transfected with wild type or dominant-negative PDEF
Expression vectors for wild type and dominant-negative PDEF
Polyclonal rabbit anti-PDEF antibody

e. Conclusions

Alterations in gene expression are central to development and differentiation of tissues, cell death, proliferation and transformation, and in the context of this grant to prostate cancer and the role of PDEF. Our progress over the last year has provided significant further evidence that PDEF is an important player in prostate cancer. We were able t demonstrate that PDEF expression is regulated by androgen and that PDEF is indeed a critical regulator of PSA gene expression in prostate cancer cells. We, furthermore, demonstrated that PDEF is significantly upregulated in a number of prostate cancer patients and, thus, possibly may serve as a marker for prostate cancer. We also showed that PDEF expression is downregulated by the growth inhibitor TGF-β1 and that PDEF itself can regulate cell proliferation. We also identified a variety of target genes for PDEF using transcriptional profiling which will further help us to understand the biological function of PDEF. Our results as well as the critical roles of other Ets factors in cellular differentiation and tumorigenesis strongly suggest that PDEF is an important regulator of prostate gland development and plays a role in prostate and breast cancer progression or development. The new data have further strengthened our believe that PDEF is a prime target for drug development.

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